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Sex hormone-binding globulin (SHBG) is a plasma protein that binds androgens. It also is a transducer of androgen signaling at the plasma membrane of prostate cancer cells. We have found that the human prostate cancer cell line, LNCaP, in addition to having a receptor for SHBG (R_{SHBG}), produces its own SHBG. We hypothesize that local regulation of SHBG production and/or secretion results in important autocrine and paracrine effects that influence gene expression and growth in prostate cancer cells.

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Progress report: "Unique Approaches to Androgen Effects on Prostate Cancer"

INTRODUCTION

Background: Androgens have two major roles in prostate cancer. First, individuals deprived of androgens early in life, such as in castration in youth or in the syndrome of 5α reductase deficiency, do not get prostate cancer. Second, once prostate cancer is established and disseminated, androgen deprivation causes temporary remission/improvement in the majority of patients so treated. These two clinical facts are not fully explained by our current understanding of how androgens exert their effects.

We have shown the plasma protein sex hormone-binding globulin (SHBG) not only binds testosterone (T) and other androgens in plasma, but is part of a prostatic androgen signal transduction system that starts with a receptor (R_{SHBG}) for SHBG on prostate cell membranes rather than the intracellular androgen receptor (AR)(1). The SHBG-R_{SHBG} complex is activated by an appropriate steroid hormone, such as estradiol (E_2) or 5α-androstan-3α, 17β-diol (3αdiol) (forming the new complex, 3αdiol-SHBG-R_{SHBG}) to trigger a second messenger system to produce cAMP within minutes of steroid binding. Furthermore, in whole non-cancerous explants of human prostate, the system can cause increases in the secretion of prostate specific antigen (PSA), an event previously thought to be related only to activation of the AR by T or dihydrotestosterone. The studies demonstrating the foregoing were based on a major assumption that SHBG (which, like most plasma proteins, is produced in the liver) arrived at the prostate only by way of the plasma. We now have shown and published that prostate cancer cells stain with anti-SHBG antibodies and, more importantly, that a number of prostate cancer cell lines (LNCaP, PC-3, and DU-145) contains both SHBG mRNA and SHBG protein. The expression of SHBG by prostate cancer cells raises the important question of how local regulation of SHBG synthesis might function either to act on the sequestration of steroid hormones within the prostate or to alter androgen induced signal transduction in an autocrine or paracrine fashion.

Objective Hypothesis: We propose that the expression of SHBG by prostate cancer cells is biologically regulated and that this SHBG functions to alter the effects of androgens and estrogens within the prostate cancer cell.

Specific Aims: (Aim 1) Generate prostate cancer cell lines that stably express SHBG in a regulatable fashion. Ascertain the (Aim 2) autocrine and (Aim 3) paracrine effects of prostate SHBG synthesis on steroid signaling. (Aim 4) Examine the effect of SHBG synthesis within the prostate on the growth of prostate cancer cell lines both in the presence and absence of androgen.

Experimental Design: We will undertake genetically and pharmacologically based studies to address our hypothesis. Prostate cancer cells from Aim 1 will be exposed to steroids that selectively stimulate either the androgen receptor or the SHBG-receptor based pathways. Further, although AR and SHBG both are high affinity binders of testosterone and DHT, there are other ligands that are specific to each. The same situation exists for

inhibitors of each of the two systems. We will independently stimulate and/or inhibit each of these two signaling systems with such ligands. This pharmacologic approach will allow us to dissect the influence of androgens on these two pathways and further ascertain how each contributes to the growth of prostate cancer cells.

BODY

We have made considerable progress in creating cell lines that overexpress SHBG, having now obtained necessary reagents for their full characterization. We have new and potentially exciting evidence that in the LNCaP cell line, SHBG is expressed as a novel isoform(s), different from the hepatically derived isoform that we have already overexpressed in LNCaP cells. We intend to further characterize the novel SHBG isoforms that we suspect are being expressed in LNCaP cells. If this latter finding extends to other prostate cell lines, normal prostate tissue and prostate tumors, it opens the exciting possibility that the prostate expresses SHBG isoforms with different functions than the hepatically derived SHBG isoform. Our studies planned for the upcoming second year of this grant will address these important questions.

1. Anti-human SHBG antibodies for Western blot analysis.
A major technical problem that we addressed this past year was identifying an antibody that would be suitable for Western blot analysis of cellular extracts. First, we tested all polyclonal and monoclonal antibodies that we have generated in house or have obtained from outside sources on HepG2 cellular extracts and purified SHBG protein from human serum. None proved useful for Western blot analysis. We next had custom polyclonal antisera generated against both the secreted form of SHBG (encoded by the cDNA used in these studies) and the alternative form of SHBG first described in the testis (2). Unique peptides, CLRPVLPTQSA and CFSLRLTHPPRTW, corresponding to the respective SHBG isoforms, were synthesized and used to immunize rabbits. Affinity purified antisera were positive by ELISA assay, however they did not prove useful for Western blot analysis. Fortunately, in 2004, we obtained a polyclonal antibody, WAK-S102-12-53 (WAK-Chemie, Steinbach, Germany), which is useful for Western blot analysis. As detailed below, we used WAK-S102-12-53 to confirm ELISA and PCR results on cell lines that inducibly or constitutively express various SHBG constructs at elevated levels.

2. Generation of LNCaP clonal cell lines that constitutively overexpress SHBG: pSHBG-FL and pSHBG-MP.
Along with the inducible LNCaP cell line, L5S2 that expresses and secretes abundant amounts of SHBG when treated with the inducing agent, PonA (see **Figure 1**), we synthesized two additional constructs for evaluating the effects of constitutively expressed SHBG in LNCaP cells. The vector used for these experiments was pCMVFlag, a plasmid containing a CMV promoter upstream of an ATG start codon, immediately followed by three iterations of a Flag tag (4) sequence. We generated, 1) pSHBG-FL, a plasmid containing the full length SHBG cDNA sequence cloned immediately downstream of the Flag tags and in the same reading frame, and 2) pSHBG-

5

MP, a plasmid containing SHBG cDNA lacking the 29 amino acid amino terminal leader sequence which, in the liver, is cleaved from the nascent protein before secretion. Thus, this Flag tagged protein mimics the mature, processed SHBG found in serum. However, since it lacks the leader sequence, we hypothesized that the SHBG-MP protein would remain inside cells. We expected that the Flag tag would allow us to specifically detect the expressed pSHBG-FL and pSHBG-MP proteins in western blots. In addition, the Flag tag would produce different sized SHBG proteins, distinguishable from endogenous SHBG.

We expected that SHBG-FL might be detectable in cells, but it would have the Flag tag cleaved along with the signal peptide prior to secretion. However, if the cleavage system was overloaded with SHBG-FL protein, we might also detect residual full length SHBG-FL protein. We expected that the SHBG-MP construct would remain inside cells because it lacked a leader sequence, and might serve to mimic the fate of absorbed SHBG from outside. The CMV promoter would direct constitutive expression of these proteins at elevated levels.

pSHBG-FL and pSHBG-MP were transfected into LNCaP cells. Following selection, 12 resistant colonies from each transfection were isolated and expanded. LNCaP-FL7 and LNCaP-MP8 were positive in Western blot analysis using anti-Flag monoclonal antibody and the anti-SHBG WAK-S102-12-53 polyclonal antibody (**Figure 2a**). LNCaP-FL7 secreted non Flag-tagged SHBG, demonstrating that the leader peptide is properly processed from Flag-tagged SHBG-FL, and that the mature processed protein is secreted just like the secreted form of SHBG in the liver. These results were confirmed by immunofluorescence staining (**Figure 2b**) and ELISA assays (data not shown). It is currently unclear why the full length SHBG cDNA, when expressed in LNCaP cells either constitutively or inducibly, is seen as two SHBG bands. This could be something intrinsic to LNCaP cells, themselves, as the construct has been verified to have the correct SHBG cDNA sequence

3. LNCaP cells express novel SHBG isoforms- structural analysis of LNCaP mRNA species.
The bulk of published data on SHBG gene expression at the mRNA level are based on work that was performed prior to the advent of PCR. It had been thought from early cDNA cloning experiments that two mRNA species are synthesized from the human SHBG gene (2-3). The first is the secreted form of SHBG, made in the liver. This 8 exon-long species is the isoform we have overexpressed above. The second SHBG isoform was originally described in the testis, and apart from a recent publication that shows it to be expressed in human sperm (5), it remains very poorly characterized. This isoform uses an upstream promoter and therefore has different first exon sequences. Early cDNA studies suggested that this isoform has exon 7 sequences spliced out.

During the past year, we obtained data suggesting that SHBG gene expression in LNCaP cells is different from the picture described above. We prepared first strand cDNA from total cellular LNCaP RNA using oligo dT primers. Using primers that amplified exon 5-8 sequences, we unexpectedly generated three RT-PCR transcripts (**Figure 3**). Sequence

analysis revealed that the largest transcript contained faithful splicing of exon 5,6,7,and 8 sequences. The intermediate transcript was missing all of exon 7 sequences. The smallest transcript was missing both exon 6 and 7 sequences.

Using primers that amplified exon 2-8 sequences, we generated four RT-PCR transcripts (data not shown). The largest transcript has a predicted size of contiguous exon 2-8 sequences. The intermediate sized transcripts are of sizes consistent with the splicing out of exon 7 and exons 6 and 7, respectively. The smallest transcript is of a size lacking exons 5, 6, and 7; however, these results are awaiting sequence analysis to confirm the size predictions. Thus, in LNCaP cells, we have evidence that at least four different SHBG mRNA species are synthesized. These mRNAs would encode proteins that differ both in size and in their carboxy terminal sequences.

We were unable to generate RT-PCR transcripts from LNCaP cells using secreted SHBG exon 1 specific primers (**Figure 4**). This could be specific to the primers used or the low abundance of SHBG mRNA in LNCaP cells (experimental in nature), or may reflect the true state of LNCaP SHBG transcripts. As expected, HepG2 RT-PCR samples were positive for the secreted SHBG exon 1 transcript (6). This is a very important finding, suggesting that a different upstream promoter is being utilized in LNCaP cells. We designed PCR primers specific for testis SHBG isoform first exon sequences and total RNA from testis as a positive control. We generated four RT-PCR bands, indicating that the testis promoter is active in LNCaP cells, and that the transcripts generated from this promoter undergo alternative splicing (data not shown). We are now in the process of sequencing the RT-PCR products so that we get a clear picture of the alternative splicing pattern.

We have also made an important and unexpected discovery that a novel, third human SHBG gene promoter exists, and is being used by LNCaP cells. The 5' ends of SHBG gene transcripts were specifically amplified from LNCaP, MCF-7, and HepG2 cells by RT-PCR, through use of the FirstChoice RLM-RACE Kit (Ambion). Briefly, this kit invokes a series of enzymatic steps designed to allow only full length, capped RNA species to undergo ligation to a synthetic RNA adaptor of known sequence. cDNA synthesis is directed by a gene-specific oligonucleotide primer, and subsequent RT-PCR analysis is performed using an upstream adaptor-specific primer, and a nested downstream gene specific primer. In these experiments, an exon 3 specific oligo was used to direct cDNA synthesis for each of the cell lines, and RT-PCR analysis was performed using a primer set that included a nested downstream exon 2 specific primer. This promoter lies approximately 17kb upstream of the promoter used for generation of the secreted form of SHBG. We have generated RT-PCR bands using an upstream primer that is specific to this promoter, and the downstream exon 8 primer (data not shown). These results have profound effects on our understanding of the structure and function of endogenously expressed SHBG, and are now the main focus of our work because, before proceeding further with our functional studies, it is necessary to have a clear picture of what SHBG isoforms are being expressed in the prostate.

4. Immunohistochemical analysis of SHBG protein expression in LNCaP cells and effects of steroid binding on SHBG localization.

Previously, we showed that LNCaP cells can be stained using anti-SHBG antibodies (7). We performed similar experiments on inducible L5S2 cells that had been stably transfected with a PonA inducible full length construct. **Figure 5** shows relative levels of SHBG in uninduced vs. induced cells, and the effects of DHT and E2 treatment on SHBG expression. We should point out that the photomigrographs shown in Figure 5 did not reproduce very well for technical reasons. From our observations, there is more pronounced staining of SHBG in the induced L5S2 cells, and pronounced staining within what appears to be the ER and/or Golgi, although this needs to be further investigated. When 2nM DHT is incubated with induced L5S2 cells for 2 or 24 hours, the cells show a rounder morphology, with slightly more focal staining. When treated with 10nM DHT, the intensity of SHBG staining in induced L5S2 cells diminishes and becomes even more focal. Induced L5S2 cells treated with E2 do not appear to show much of a staining difference when compared to untreated induced cells.

We have purposely delayed our functional analyses and are devoting much effort to what we consider a highly important aspect of our study, namely characterizing the new SHBG mRNA transcripts that we have discovered in LNCaP cells. There is a strong possibility that an SHBG isoform other than the hepatically secreted protein is synthesized in LNCaP cells. We think it is prudent to first ascertain the exact SHBG expression profile so that we can accurately proceed with our functional studies. The structures of these species should tell us whether the proposed studies mimic only the effects of serum-derived SHBG on prostate cells, or include the effects of endogenously synthesized SHBG.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of the anti-human SHBG polyclonal antibody, WAK-S102-12-53, for use in Western blot analysis.
- Synthesis of pSHBG-FL and pSHBG-MP, flag tagged full length and mature processed SHBG constructs for constitutive expression. Generation of candidate LNCaP-FL7 and LNCaP-MP8 clonal cell lines that constitutively expresses stably incorporated flag tagged full length and mature processed SHBG construct, respectively.
- Demonstration that elevated amounts of SHBG can be made in LNCaP cells, and that LNCaP overexpressing cells secrete SHBG. Demonstration that the flag tagged mature processed SHBG protein is stable and remains intracellular.
- LNCaP cells express multiple SHBG mRNA species, including forms that retain exons 2-8, and those that splice out exons 7, 6+7, and perhaps 5, 6, and 7. RT-PCR results suggest LNCaP cells express novel SHBG isoforms that lack the exon 1 sequences present in hepatically derived SHBG. LNCaP cells appear to use the testis promoter and a novel upstream promoter.

REPORTABLE OUTCOMES:

1. Fourth International Symposium on Hormonal Carcinogenesis. Valencia, Spain, June 21-25, 2003
Poster presentation and book chapter:
IMMUNOHISTOCHEMICAL AND *IN SITU* DETECTION OF SEX HORMONE-BINDING GLOBULIN (SHBG) EXPRESSION IN BREAST AND PROSTATE CANCER: IMPLICATIONS FOR HORMONE REGULATION

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2. Third International Meeting- Rapid Responses to Steroid Hormones. Florence, Italy, Sept. 12-14, 2003
Poster presentation:
IMMUNOHISTOCHEMICAL AND *IN SITU* DETECTION OF SEX HORMONE-BINDING GLOBULIN (SHBG) EXPRESSION IN BREAST AND PROSTATE CANCER: IMPLICATIONS FOR HORMONE REGULATION

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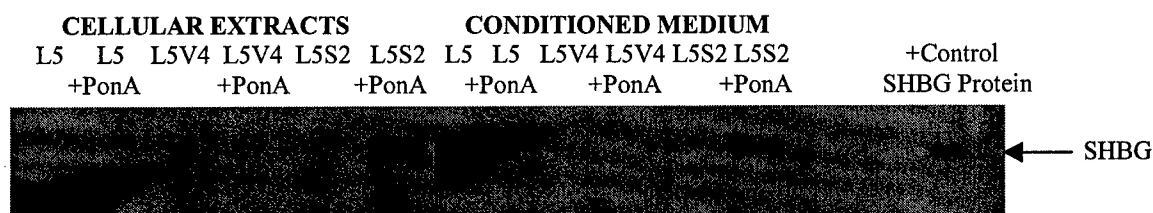
CONCLUSIONS:

We have shown that LNCaP cells can be used to produce the hepatically secreted isoform of SHBG. From our initial RT-PCR results, we expected that this was the major isoform expressed in prostate cells. However, based on our newer RT-PCR results, this may not be the case. Alternative splicing and alternative promoter utilization appear to be responsible for generating at least four different SHBG transcripts in LNCaP cells. At this time, we are characterizing the structures of these transcripts. If we confirm that a novel SHBG isoform(s) is expressed in LNCaP cells and in normal prostate epithelial cells in general, this could change our prior hypothesis of how allelic deletions of the SHBG gene locus could contribute to prostate cancer. Now that we have a useful polyclonal antibody for Western blot analysis, we will be able to address these and other questions. If novel isoforms are the endogenously expressed species in the prostate, we will use a similar overexpression and downregulation strategy to ascertain their functions, and we will approach our original strategy more as a model for the effects of serum SHBG on the prostate.   In summary, the results from our first year of study reveal that SHBG expression in the prostate is more complicated than previously thought, consisting of multiple transcripts that encode different isoforms. In year 2, we anticipate completing our characterization of these novel transcripts, and addressing their functionality.

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APPENDICES:
Figure 1. SHBG overexpression is induced by PonA in L5S2 cells

| CELLULAR EXTRACTS						CONDITIONED MEDIUM						
L5	L5 +PonA	L5V4	L5V4 +PonA	L5S2	L5S2 +PonA	L5	L5 +PonA	L5V4	L5V4 +PonA	L5S2	L5S2 +PonA	+Control SHBG Protein

← SHBG

Figure 1: PonA induces SHBG protein expression in LNCaP cells stably transfected with pVgRXR (a plasmid encoding the PonA-activatable transactivator), and the inducible construct, pIND/Hygro/SHBGsense (a plasmid that expresses the secreted form of SHBG in response to the activated transactivator). Parental L5, vector control L5V4, and inducible L5S2 cells were plated in duplicate in 6 well dishes at 75% confluence. After incubating for 48 hours, cells in one well were exposed to 10uM PonA for 24 hours, while control, unexposed cells were mock treated with solvent. Total cellular protein was prepared, and conditioned medium was isolated and spun down to remove cells and cellular debris. Cellular extracts and 30ul aliquots of conditioned medium were analyzed by Western blot. 10ng of purified SHBG was loaded in the positive control lane. This short exposure shows inducible expression of SHBG in L5S2 cells, and secretion. Two SHBG bands are visible in the induced L5S2 lanes, similar findings were obtained using a constitutively expressed SHBG full length construct in LNCaP cells (see Figure 2). The reason for this observation is unclear at the present time.

Figure 2. Western blot and immunofluorescence analysis of SHBG overexpression in LNCaP clonal cell lines

Cellular Extracts   Conditioned
Medium
HEPG2 LNCaP LMP8 LFL7 LNCaP LMP8 LFL7
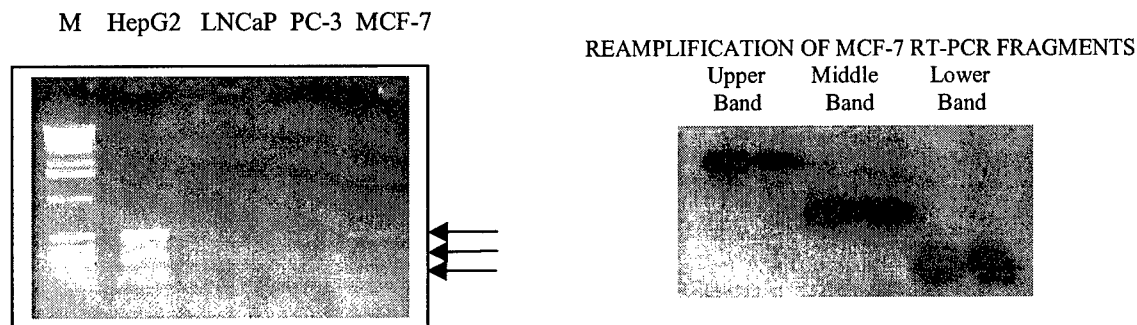

Flag tagged SHBG-MP (46kD)→
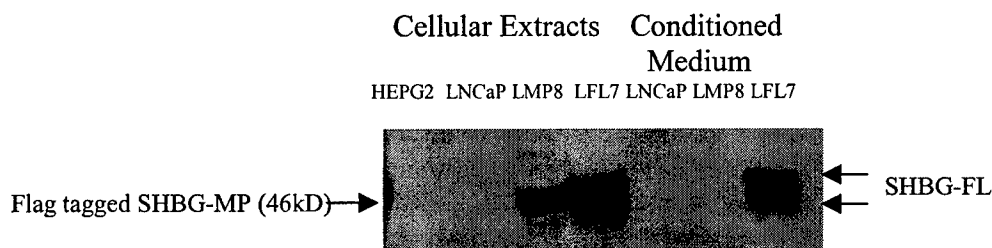
← SHBG-FL
←

Figure 2a: Ectopic expression of Flag-tagged SHBG-MP and Flag-tagged SHBG-FL in stable LNCaP clones. Total cellular protein extracts were prepared from parental LNCaP cells, and clonal LMP8 and LFL7 cells (transfected with the FLAG tagged SHBG MP and FL constructs, respectively), as well HEPG2 cells. Conditioned medium was isolated from parental LNCaP and clonal LMP8 and LFL7 cells, and centrifuged to remove cells and cellular debris. Ectopically expressed and secreted SHBG was detected by Western blot analysis, shown above, using the anti-human SHBG WAK-S102-12-53 antibody.

A: LNCaP MP-8   B: LNCaP FL-7   C: Parental LNCaP cells
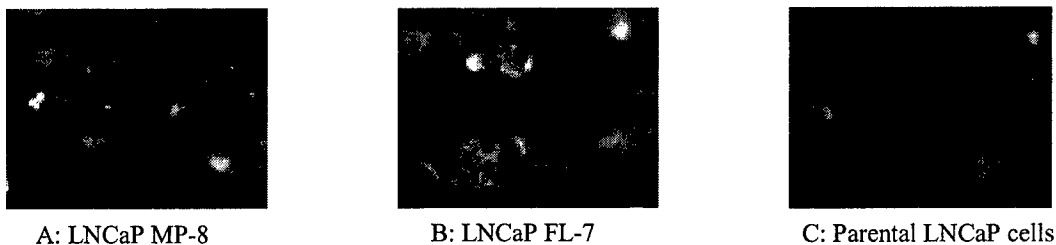
Figure 2b. Immunostaining of stable LNCaP clones that constitutively express a mature processed SHBG or a full length SHBG construct. LNCaP MP-8, FL-7, and parental cells were plated on glass slides, fixed, and exposed to an anti-human SHBG polyclonal antibody. They were developed with a rabbit anti-mouse IgG1 linked to the green fluor, Alexa-488. Relative SHBG staining is shown (parental cells exhibited relatively low staining).

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Figure 3. SHBG is alternatively spliced: RT-PCR analysis of LNCaP cells.

M   HepG2   LNCaP   PC-3   MCF-7

REAMPLIFICATION OF MCF-7 RT-PCR FRAGMENTS

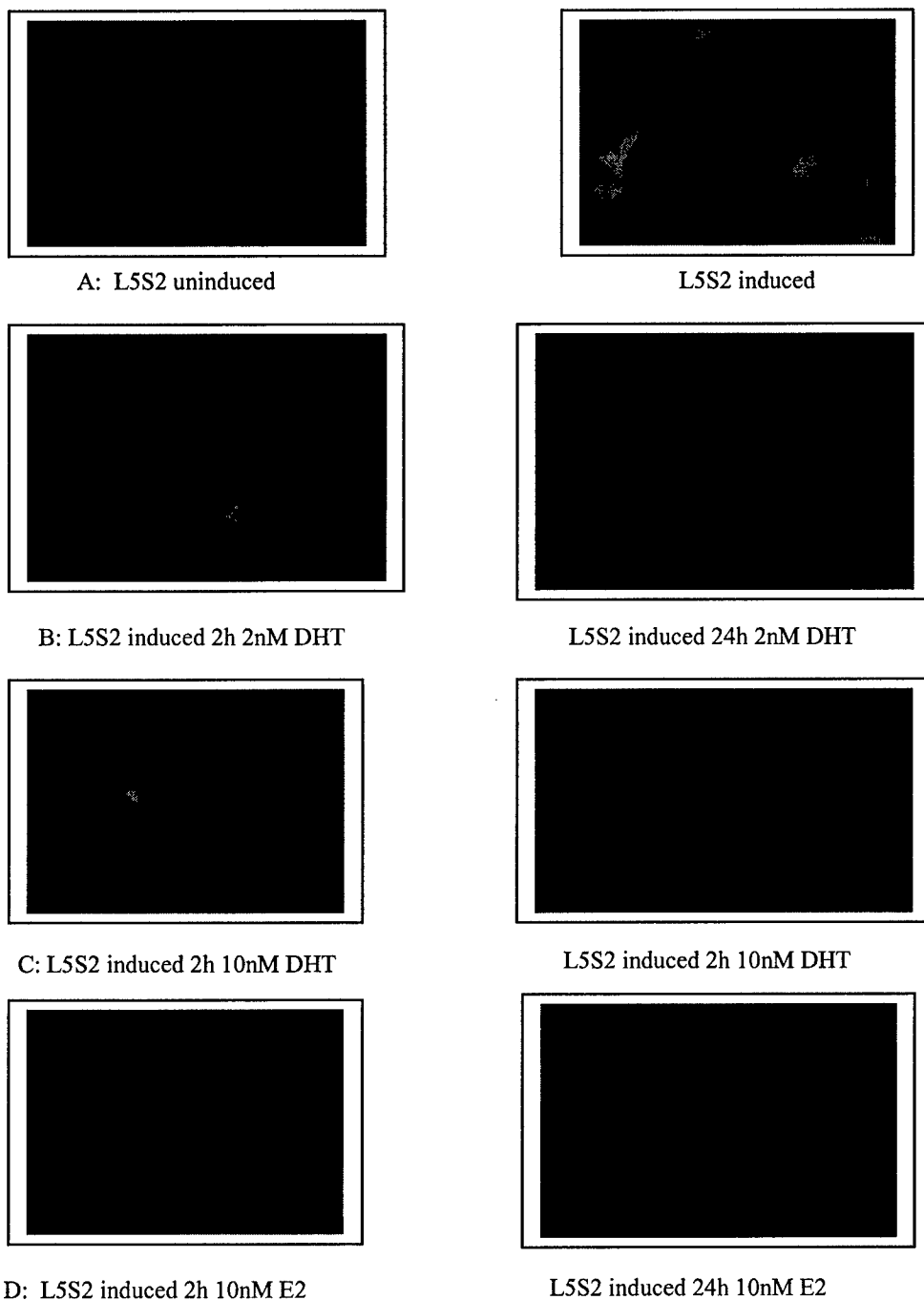| Upper Band	Middle Band	Lower Band

Figure 3. RT-PCR analysis reveals alternative splicing in LNCaP cells. Left: Total cellular mRNA was prepared from exponentially growing LNCaP, HepG2 (a human hepatic cell line that secretes SHBG and was included as a positive control), MCF-7, and PC3 cells. First strand cDNAs were generated using an oligo dT primer. PCR was then performed using primers predicted to amplify exon 5-8 sequences. Three RT-PCR transcripts were generated in all samples, the predicted 521nt band, and two smaller bands (three light LNCaP bands (arrows) were visible in the original gel). We have reproduced this alternative splicing pattern three times, using different total cellular RNA preparations. Right: MCF-7 RT-PCR products were electrophoresed alone in a single gel (not shown). DNA from each MCF-7 band was reamplified using the same exon 5 and 8 primers. Duplicate lanes were run for each sample (right), and DNA was extracted from the gel. DNA sequence analysis showed the 521nt band to contain contiguous exon 5-8 sequences. Similar results were obtained for LNCaP cells (data not shown). The middle band is missing all of exon 7, and the small band is missing exons 6 and 7. M: DNA size marker.

Figure 4. LNCaP cell SHBG mRNAs do not contain the hepatically secreted isoform exon 1 sequences.

Marker   1   2   3   4   5   6   LNCaP MCF-7
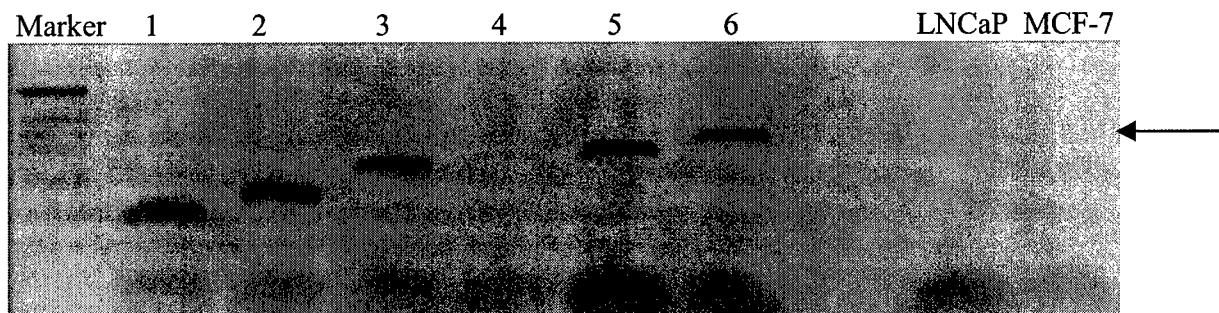
Figure 4. RT-PCR analysis of SHBG exon 1 sequences. The same first strand cDNA samples from Figure 3 were amplified by PCR. Control HepG2 RT-PCR amplifications were performed using a single exon 1 forward primer and six different exon 2 (lanes 1 and 2) or exon 3 (lanes 3-6) reverse primers in order of increasing fragment size. LNCaP and MCF-7 RT-PCR amplifications were performed using the same exon 1 forward and a single exon 3 reverse primer (the same set as in HepG2 lane 6). RT-PCR transcripts of expected sizes are present in all HepG2 amplifications. The exon 1-3 RT-PCR fragment is absent in MCF-7 and LNCaP cells (arrow).

Figure 5. Immunohistochemical detection of SHBG in inducible LNCaP cells-steroid effects on localization

A: L5S2 uninduced

L5S2 induced

B: L5S2 induced 2h 2nM DHT

L5S2 induced 24h 2nM DHT

C: L5S2 induced 2h 10nM DHT

L5S2 induced 2h 10nM DHT

D: L5S2 induced 2h 10nM E2

L5S2 induced 24h 10nM E2

Figure 5. Immunohistochemical detection of SHBG in inducible L5S2 cells, and effects of DHT and E2 treatment. L5S2 cells were plated on glass slides, induced with Pon A (where indicated), treated with the indicated steroid for either 2 or 24 hours, and then fixed, and exposed to an anti-human SHBG polyclonal antibody. They were developed with a rabbit anti-mouse IgG1 linked to the green fluor, Alexa-488. Relative SHBG staining is shown (control cells exhibited very low level staining). DHT treated cells displayed a rounder morphology with a more focal SHBG staining. Cells not treated with steroid, and E2 treated cells displayed a slightly stronger, cytoplasmic staining.